Iranian Journal of Veterinary Science and Technology

Received: 2019- May- 06
Accepted after revision: 2019- Jun- 16
Published online: 2019- Jul- 27

RESEARCH ARTICLE

DOI: 10.22067/veterinary.v1i11.80546

Experimental infection of pheasants with a velogenic chicken isolate of Newcastle disease virus

Ramezan Ali Jafari[a], Anahita Rezaie[b], Zahra Boroomand[a], Mansoor Mayahi[a], Reza Zare[c]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran [b] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran [c] Postgraduate student of Avian Medicine, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

ABSTRACT

Newcastle disease (ND) is a highly contagious infection of many avian species, causing enormous losses in poultry production worldwide. The objective of this study was to reveal the clinical feature, virus shedding, and immune response following infection with a velogenic chicken isolate of Newcastle disease virus (NDV) in susceptible and vaccinated pheasants. Eighty day-old pheasant chicks were allotted to four groups. At 30 days of age, the birds in groups 1 and 3 were vaccinated with B1 strain via eye drop. Two weeks later, each bird in groups 1 and 2 was inoculated with 100 µL (50 µL/eye) of NDV-infected allantoic fluid containing 10^5 EID_{50} of viral inoculum. All groups were inspected daily for three weeks. Swab samples were taken at different time points, and verified for NDV infection by using reverse-transcription polymerase chain reaction (RT-PCR). Serological examination was also made by haemagglutination-inhibition assay. Clinically, watery mucoid feces was observed only in one case among the vaccinated challenged birds, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation. Out of 20 birds in group 2, one case (5%) died. Based on RT-PCR, virus shedding was only observed among the unvaccinated birds from 5 to 14 days after challenge. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The infected birds showed a high seroconversion. In conclusion, the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and ocular vaccination with B1 strain could provide a good protection.

Keywords

Immune response, Newcastle disease, Pheasant, Virus shedding

Abbreviations

ND: Newcastle disease
NDV: Newcastle disease virus
EID_{50}: 50% embryo infective dose
HI: hemagglutination-inhibition
RT-PCR: reverse-transcription polymerase chain reaction
cDNA: complementary DNA
bp: base pairs

Corresponding author:
Ramezan Ali Jafari,
Tell/Fax: +98916 618 0976

Email: Jafari.ramezanali@scu.ac.ir

Introduction

Newcastle disease (ND) is a highly contagious and fatal disease affecting at least 241 species of domestic and wild birds of both sexes and all age groups [1]. The causative agent is a virulent virus of the avian paramyxovirus serotype I of *Avulavirus* genus belonging to the family *Paramyxoviridae* [2]. Velogenic Newcastle disease virus (NDV) is endemic in many countries of the Middle East, Africa and Asia [3]. The inclusion of ND in the list of notifiable diseases by World Organization for Animal Health (2008) is indicative of its paramount economic impact on the worldwide poultry industry [2]. Among poultry, chickens are the most susceptible, showing the most clinical signs, and ducks are the least susceptible, showing the least clinical signs. Nonvaccinated pheasants are highly susceptible with clinical signs similar to those observed in chickens [3].

The first outbreaks of ND were observed in chickens in 1926 in Java, Indonesia, and Newcastle-upon-Tyne, England [3]. Then, NDV was spread throughout the world, and affected other avian species such as turkeys and quails [4,5]. Several outbreaks of ND have been also reported in pheasants in East Anglia [6], Iraq [7], Great Britain [8], Denmark [9], and South East England [10].

In Iran, NDV is endemic in different parts of the country, causing enormous losses due to high mortality, sub-optimal production, slaughterhouse condemnation of carcasses, and high prevention and treatment expenses. In recent years, outbreaks of ND have been occasionally observed in different avian species in Iran, including Japanese quail [11], ostrich [12], exotic caged birds [13], and broiler chickens [14]. During 2012-2013, some outbreaks with heavy losses occurred among commercial broiler chicken flocks located in southwest Iran. The isolated viruses were classified as genotype VII, and subsequently to subgenotype VIId [15].

Today, pheasant is extensively reared in several countries of the world as a game bird or for the purpose of human consumption. In recent years, commercial production of pheasant has increased in some regions of Iran, and a part of protein demands of Iranian people is provided with the meat of this bird. The NDV may be easily transmitted from one avian species to another, and some outbreaks in pheasants were epidemiologically related to the spread of the virus from chickens [6] and feral migratory birds [9]. Considering that viruses emerging from field strains may possess relatively new features, this study was conducted to investigate the clinical manifestations, virus shedding and serological responses following infection with a velogenic chicken isolate of NDV in susceptible and vaccinated pheasants.

Results

Clinical signs

No morbidity or mortality was observed in unchallenged pheasants. The vaccinated challenged birds exhibited watery mucoid feces only in one case, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation (Figures 1 and 2). Almost one-fourth of these birds became morbid, but 1 (5%) out of 20 pheasants died at 10 days postinoculation. Clinical symptoms appeared 7 days postinoculation and continued by 12 days after challenge.

Virus shedding

An attempt to detect the virus was made for a period of three weeks, and the results of the PCR are presented in Figure 3. Out of 10 birds in group 2 which were sampled after challenge, 6 cases (60%) shed the NDV through respiratory and/or intestinal tracts from 5 to 14 days postinoculation. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The tracheal swabs were virus-positive at 5 and 10 days post-challenge, but the cloacal swabs were virus-positive at 10 and 14 days. The NDV was not detected in samples obtained from the other groups (Table 1).

Serological examination

The antibody response of pheasants to vaccination and or challenge with velogenic NDV is summarized in Table 2. The HI titers of the serum samples of all groups were negative (i.e., $< Log_2 3$) before vaccination. This status continued in the birds of group 4 by the end of the experiment, whereas a significant seroconversion occurred after vaccination or challenge in the other groups ($p < 0.05$). In group 3, the HI titer increased significantly after vaccination ($p < 0.05$), but its change was not significant from 14 to 28 days postvaccination ($p > 0.05$). After challenge, the HI titers increased in groups 1 and 2; although its elevation was only significant ($p > 0.05$) in group 2.

Discussion

The NDV continues to be a major threat to the poultry industry. After infection with velogenic NDV, the nonvaccinated birds may die suddenly with no clinical signs and with a death rate of 100% [3], although the severity of the disease observed with any given virus greatly varies depending on host parameters, including species, breed, age and immune status,

Figure 1
Depression in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (7 days postinoculation).

Figure 2
Head deviation in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (8 days postinoculation).

Figure 3
Electrophoresis of RT-PCR product of F gene in pheasants inoculated with a velogenic chicken isolate of Newcastle disease virus; M: ladder (100 bp), N: negative control, P: positive control (330 bp), lanes 1 - 5: positive samples.

Table 1
Virus shedding in pheasants experimentally infected[1] with a velogenic chicken isolate of Newcastle disease virus.

Group	Swab	Days postinoculation[2]					
		0	2	5	10	14	21
Vaccinated and challenged	Tracheal	-	-	-	-	-	
	Cloacal	-	-	-	-	-	
Unvaccinated and challenged	Tracheal	-	-	2	2		
	Cloacal	-	-	-	1	2	
Vaccinated and unchallenged	Tracheal	-	-	-	-	-	
	Cloacal	-	-	-	-	-	
Unvaccinated and unchallenged	Tracheal	-	-	-	-	-	
	Cloacal	-	-	-	-	-	

[1]At 30 days of age, pheasants in vaccinated groups received B1 strain vaccine via eye-drop. Two weeks later, each bird in challenged groups was inoculated through ocular route with 10^5 EID_{50} of viral inoculum.
[2]At each time point, two birds per group were sampled and examined for NDV infection by RT-PCR

Table 2
Haemagglutination-inhibition titers (Log2)[1] in pheasants following vaccination with B1 strain and or challenge with a velogenic chicken isolate of Newcastle disease virus.

Group	Days after vaccination		
	0[2]	14[3]	28[4]
Vaccinated and challenged	1.4 ± 1.06 [c]	4.8 ± 0.71 [b]	5.6 ± 0.74 [b]
Unvaccinated and challenged	1.3 ± 1.04 [c]	1.1 ± 0.83 [c]	7.1 ± 0.88 [a]
Vaccinated and unchallenged	1.3 ± 1.04 [c]	4.5 ± 0.93 [b]	4.8 ± 0.96 [b]
Unvaccinated and unchallenged	1.8 ± 1.04 [c]	1.3 ± 1.05 [c]	0.9 ± 0.83 [c]

[a-c] Values within columns/rows with no common superscripts differ significantly ($p < 0.05$).
[1] Values represent Means ± SE calculated from eight birds per group at each time point.
[2] Thirty days of age, [3] Before challenge, [4] Two weeks after challenge.

coinfection with other organisms, environmental and nutritional conditions, and route of exposure [1,16]. The virus used in this study was isolated from an outbreak of ND in a vaccinated chicken flock with a high mortality, and was characterized as a velogenic strain based on the sequencing of the F protein cleavage site. However, in this experiment, only one of 20 birds in group 2 died and the other morbid birds recovered rapidly. To our knowledge, there is no information regarding the experimental pathogenicity of NDV for pheasants to be compared with our results. But, the lower severity of the disease observed in pheasants can be associated with their less susceptibility, as well as route of infection (i.e. experimental vs natural) [2,10]. In a similar study performed by Wakamatsu et al. (2006), chickens challenged with velogenic NDV exhibited clinical sings at 2 days after inoculation and experienced a mortality rate of 100%, whereas commercial turkeys developed the clinical disease later (i.e., 6 days after inoculation) with no mortality. [16]. In the ND outbreaks in Italy, Capua et al (2000) reported that chickens and Guinea fowl were the most affected species, followed by pheasants, turkeys and ostriches [17]. In the current study, the vaccination of pheasants with B1 strain did not cause any clinical signs associated with post-vaccinal reactions. Similarly, Schmidt et. al. (2008) didn't find any reactions in pheasants vaccinated via eye-drop with B1, Ulster 2C, or LaSota starins [18]. The general clinical signs, including anorexia, lethargy, and head deviation observed in the unvaccinated challenged birds are the typical of the disease. They were all reported previously in natural infections of pheasants with NDV [6,7,17,19].

RT-PCR is one of the reliable laboratory techniques facilitating a rapid diagnosis by detecting NDV virus in clinical specimens [2]. In the current study, NDV was first detected in tracheal swabs obtained from nonvaccinated challenged pheasants at 5 days postinoculation before the appearance of the clinical signs (i.e., 7 days after inoculation). In our previous work, the Wishard bronze poults experimentally infected with highly virulent NDV of chicken isolate shed the virus 2 days earlier than the exhibition of clinical signs [20]. Wakamatsu et al. (2006) isolated NDV from swab samples of infected turkeys at 2 days postinoculation, whereas the onset of the disease was at 6 days postinoculation [16]. Moreover, in the present study, the tracheal swabs had a higher virus detection rate (4/10), compared to the cloacal swabs (3/10). This is somewhat consistent with the results reported by the previous studies, in which NDV was more frequently isolated from oral swabs than from cloacal swabs [16,21]. The detection or isolation rate of NDV may be influenced by the tropism of the virus. In an experimental study performed by Perozo et al. (2008), VG/GA strain of NDV was detected more in samples obtained from the intestinal tract of broiler chicks, whereas LaSota strain was detected more in samples taken from respiratory tracts [22]. Nevertheless, some studies have demonstrated the lack of sensitivity in detecting the virus in fecal samples, because they contain more extraneous organic material that can interfere with RNA recovery and amplification by PCR, suggesting that tracheal or oropharyngeal swabs are often the specimens of choice [2]. In the present study, virus shedding was not observed in vaccinated challenged pheasants. This is likely to be associated with less sensitivity of virus detection than isolation method, the small number of birds sampled at any time point after challenge, and or less susceptibility of pheasants to NDV in comparison with chickens, however additional studies are needed to clearly explain this finding.

As shown in Table 2, the HI titer in group 4 was lower than $\log_2 3$ before vaccination, and remained constant during the experiment, which can be regarded as being nonspecific [2]. On the other hand, a sudden seroconversion was observed in the other groups, implying the induction of active immunity by vaccinal or challenging virus. In group 3, mean HI titers of 4.5-4.8 (Log_2) were resulted from vaccination, but did not change significantly during 14 to 28 days postvaccination. In a similar study performed by Schmidt et. al. (2008), ocular vaccination of 10-day-old pheasants with B1, Ulster 2C, or LaSota produced mean HI titers of 4.2-5.0 (Log2) at 24 days of age [18]. In group 2, a higher HI titer (7.1) was found after challenge by velogenic NDV. Aldous et al. (2007) reported HI titers of 2^4-2^8 (tested with 8 HAU of antigen) in pheasants suffering from an outbreak of ND [10]. Piacenti et al. (2006) found a significant seroconversion at 10 days after infection of velogenic NDV in commercial turkeys [23]. In group 1, challenge with velogenic NDV could not make a significant rise in the HI titer, which may be associated with the interference of active antibodies with velogenic NDV. Alexander and Senne (2008) reported that eye-drop vaccination of chickens with Hitchner B1 will result in production of lachrymal IgM, IgG, and IgA due to the replication of virus in the Harderian gland, which could be prevented by the presence of maternal IgG in lachrymal fluid [24]. These findings indicate that serum antibody alteration in pheasants following exposure to NDV is rapid and very similar to that in chickens and turkeys. In infection of chickens with NDV, antibodies usually are detectable in the serum within 6-10 days and reach the peak after 2-4 weeks [22,24].

In conclusion, the results indicated that the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and that ocular vaccination with B1 strain along with biosecurity could provide a good protection.

Material and methods

The velogenic NDV used in this experiment was isolated from a broiler chicken flock in southwest Iran during an outbreak in 2013. Based on nucleotide sequence, the virus was previously characterized as genotype VII (subgenotype VIId), and assigned an accession number of NDa:KP347437 [15]. Initially, the virus was propagated twice in 9-day-old embryonated chicken eggs through inoculation into chorioallantoic sac. The 50% embryo infective dose (EID_{50}) was calculated for the second passage according to the method of Reed and Muench [25], and the harvested allantoic fluid was used as inoculum as specified in the experimental design.

Experimental Design

A total of 80 day-old unsexed pheasant chicks were purchased and randomly assigned into four equal groups. They were housed in cages separately in the Animal Research Unit of Shahid Chamran University of Ahvaz, and received feed and water ad libitum during the experiment. At 30 days of age, when the sera were negative for maternal antibodies in conventional hemagglutination-inhibition (HI) test, the birds in groups 1 and 3 were vaccinated with live B1 strain of NDV via eye drop; but those in groups 2 and 4 were sham-vaccinated with distilled water. Two weeks later, each bird in groups 1 and 2 was inoculated with 100 µL (50 µL/eye) of NDV-infected allantoic fluid containing 10^5 EID_{50} of viral inoculum, whereas the birds in groups 3 and 4 received distilled water by the same route. All birds were daily inspected for clinical manifestations and mortality for three weeks.

Sample collection

Tracheal and cloacal swabs were obtained from two birds per group before inoculation and at 2, 5, 10, 14, and 21 days postinoculation. They were examined for NDV infection by reverse-transcription polymerase chain reaction (RT-PCR). Moreover, eight birds from each group were bled through jugular vein before vaccination and at 14, and 28 days postvaccination. The blood samples were left to coagulate at room temperature for 8 h, and then centrifuged at 2000 rpm for 5 min. The collected sera were stored at -20°C until examined by HI test.

Reverse-transcription polymerase chain reaction

The swab samples were individually placed in microtubes containing 250 µl phosphate-buffered saline (PBS). After removing them from the microtubes, the RNA extraction was performed using the RNX™-Plus Kit (CinaGen, Tehran, Iran) according to the manufacturer's protocol. The isolated RNAs were directly used for the RT-PCR or stored at -70 °C. The partial F gene, including the cleavage site sequence, was amplified using a pair of specific primers. The primer sequences were TT GAT GGC AGG CCT CTT GC and GG AGG ATG TTG GCA GCA TT [14]. The complementary DNA (cDNA) was synthesized using BioNeer RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's instruction. The RT-PCR assay was carried out in a 20 µL reaction volume consisting of 2 µL of 10x PCR buffer, 0.2 µL of 10 mM dNTPs, 1 µL of each primer (20 pmol/ml), 0.2 µL Taq DNA polymerase (5U/ml), 0.6 µL of 50 mM magnesium chloride, 10 µL distilled water, and 5 µL cDNA dilution. The RT-PCR conditions included initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. The RT-PCR products with 330 base pairs (bp) were subjected to electrophoresis using 1.5% agarose gel. The NDV-infected allantoic fluid from our previous work was used as positive control, and distilled water was employed as negative control. In addition, a 100-bp DNA marker was used in electrophoresis for determining the RT-PCR product size.

Haemagglutination-inhibition test

The sera obtained by centrifugation of the samples were left in a water bath at 56°C for 30 min. Then, they were assessed for haemagglutination-inhibiting antibodies using 4 HA units of NDV antigen and two-fold serum dilutions as recommended by Thayer and Beard (2008) [26]. The results were expressed as Log2.

Statistical analysis

All data were analyzed in SPSS software (Version 24.0., Armonk, NY: IBM Corp.) using one-way analysis of variance. Differences showing $p<0.05$ were considered statistically significant [27]

.

Acknowledgments

The authors would like to express their gratitude to Vice-chancellor for Research of Shahid Chamran University of Ahvaz (Ahvaz, Iran) for financial support of this work, and to Alireza Kazemi for preparation of Figure 3.

Author Contributions

Conceived and designed the experiment: RAJ, AR, ZB, Consulted: MM, Performed the experiment: RAJ, AR, ZB, RZ, Wrote the paper: RAJ, RZ.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

1. Kaleta EF, Baldauf C. Newcastle disease in free living and pet

birds. In: Alexander DJ, editor. Newcastle disease. Netherlands: Kluwer Academic Publishers; 1988.

2. World Organization for Animal Health (OIE). Newcastle disease. In: Manual of diagnostic tests and vaccines for terrestrial animals. 6th ed. OIE, France; 2008.

3. Miller PJ, Koch G. Newcastle disease. In: Swayne DE, Glisson JR, McDougald LR, Nolan LK, Saurez DL, Nair V, editors. Diseases of poultry. 13th ed. Iowa: Blackwell publishing;2013.

4. Gale C, McCartiney C, Sanger C. Newcastle disease in turkeys. J Am Vet Med Assoc. 1961;139:462-5.

5. Gowthaman V, Singh SD, Barathidasan R, Ayanur A, Dhama K. Natural outbreak of Newcastle disease in turkeys and Japanese quails housed along with chicken in a multi–species poultry farm in northern India. Adv Anim Vet Sci. 2013;1(3S):17-20.

6. Borland ED. Newcastle disease in pheasants, partridges and wild birds in East Anglia, 1970-1971. Vet Rec. 1972;90(17):481-2.

7. Jaafir NAH, Hatem HK, Faiza IZ, Ahalm AH. An outbreak of Newcastle disease in a pheasant flock in Iraq. Avian Pathol. 1980;9(4):583-5.

8. Alexander DJ, Manvell RJ, Frost KM, Pollitt WJ, Welchman D, Perry K. Newcastle disease outbreak in pheasants in Great Britain in May 1996. Vet Rec. 1997;140(1):20-2.

9. Jorgensen PH, Handberg KJ, Ahrens P, Hansen HC, Manvell RJ, Alexander DJ. An outbreak of Newcastle disease in free-living pheasants (Phasianus colchicus). J Vet Med Series B. 1999;46(6):381-7.

10. Aldous EW, Manvell RJ, Cox WJ, Ceeraz V, Harwood D, Shell W, et al. An outbreak of Newcastle disease in pheasants (Phasianus colchicus) in south-east England in July 2005. Vet Rec. 2007;160(14):482-4.

11. Momayez R, Gharahkhani P, Pourbaksh SA, Toroghi R, Shoushtari AH, Banai M. Isolation and pathogenicity identification of avian paramyxovirus serotype 1 (Newcastle disease) virus from a Japanese quail flock in Iran. Arch Razi Inst. 2007;62(1):39-44.

12. Ghiamirad M, Pourbakhsh A, Keyvanfar H, Momayaz R, Charkhkar S, Ashtari A. Isolation and characterization of Newcastle disease virus from ostriches in Iran. Afr J Microbiol Res. 2010;4(23):2492-7.

13. Madadgar O, Karimi V, Nazaktabar A, Kazemimanesh M, Ghafari MM, Azimi Dezfouli SM. A study of Newcastle disease virus obtained from exotic caged birds in Tehran between 2009 and 2010. Avian Pathol. 2013;42(1):27-31.

14. Mehrabanpour MJ, Khoobyar S, Rahimian A, Nazari MB, Keshtkar MR. Phylogenetic characterization of the fusion genes of the Newcastle disease viruses isolated in Fars province poultry farms during 2009-2011. Vet Res Forum. 2014;5(5):187-91.

15. Boroomand Z, Jafari RA, Mayahi M. Molecular characterization and phylogenetic study of the fusion genes of Newcastle disease virus from the recent outbreaks in Ahvaz, Iran. Virusdisease. 2016;27(1):102-5.

16. Wakamatsu N, King DJ, Kapczynski DR, Seal BS, Brown CC. Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002-2003. Vet Pathol. 2006;43(6):925-33.

17. Capua I, Dalla PM, Multinelli F, Marangon S, Terregino C. Newcastle disease outbreaks in Italy during 2000. Vet Rec. 2002;150(18):565-8.

18. Schmidt EMDS, Paulillo AC, Filho TF, Agustini M, Ventura HLB, Junior LD. Clinical and immunological parameters of Newcastle disease vaccination in juvenile ring-neck pheasants. Int J Poult Sci. 2008;7(3):283-4.

19. Kinde H, Hullinger PJ, Charlton B, McFarland M, Hietala SK, Velez V, et al. The isolation of exotic Newcastle disease (END) virus from nonpoultry avian species associated with the epidemic of END in chickens in southern California: 2002–2003. Avian Dis. 2005;49(2):195-8.

20. Jafari RA, Boroomand Z, Rezaie A, Mayahi M, Nejati Saravi A. Experimental infection of turkeys with a virulent Newcastle disease virus isolated from broiler chickens. Arch Razi Instit. 2019;74(1):51-7.

21. Haque MH, Hossain MT, Islam MT, Zinnah MA, Khan MSR, Islam MA. Isolation and detection of Newcastle disease virus from field outbreaks in broiler and layer chickens by reverse transcription-polymerase chain reaction. Bangl J Vet Med. 2010;8(2):87-92.

22. Perozo F, Villegas P, Dolz R, Afonso CL, Purvis LB. The VG/GA strain of Newcastle disease virus: mucosal immunity, protection against lethal challenge and molecular analysis. Avian Pathol. 2008;37(3):237-45.

23. Piacenti AM, King DJ, Seal BS, Zhang J, Brown CC. Pathogenesis of Newcastle disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. Vet Pathol. 2006;43(2):168-78.

24. Alexander DJ, Senne DA. Newcastle disease. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan NK, Swayne DE, editors. Diseases of poultry. 12th ed. Iowa: Blackwell publishing;2008.

25. Villegas P. Titration of biologicacal suspensions. In: Dufor-Zavala L, Swayne DE, Glison JR, Pearson JE, Reed MW, Woolcock PR, editors. A laboratory manual for the isolation, identification and characterization of avian pathogens. 5th ed. Athene, GA: American Association of Avian Pathologist; 2008.

26. Thayer SG, Beard CW. Serologic procedure. In: Dufor-Zavala L, Swayne DE, Glison JR, Pearson JE, Reed MW, Woolcock PR, editors. A laboratory manual for the isolation, identification and characterization of avian pathogens. 5th ed. Athene, GA: American Association of Avian Pathologist; 2008.

27. Petrie A, Watson, P. Statistics for Veterinary and Animal Science. 2nd ed. Iowa: Blackwell Publishing; 2006.